Locusta migratoria (Orthoptera: Acrididae) embryos monitor neighboring eggs for hatching synchrony

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Abstract

The mechanism for controlling hatching from egg masses has received little attention in insects. In this study, both the pattern of hatching and factors influencing hatching were examined for the egg mass of the migratory locust, *Locusta migratoria*, under continuous illumination at 30°C. The eggs hatched simultaneously from the egg pods with a mean hatching period of 2.4 h. When the eggs were kept in different-sized masses, they tended to hatch earlier and across a shorter period as the mass size increased. However, the eggs in each mass hatched in synchrony, irrespective of the mass size. The eggs separated from the pods, and kept singly in moist sand, hatched later and across a longer period than those kept in the pods. Egg separation performed at various times revealed that hatching time and synchrony were determined on the day prior to hatching. The same conclusion was drawn when the eggs separated on day 10 were grouped as either egg masses or pairs at various times before hatching. Two eggs from different pods, incubated in physical contact with each other, hatched in synchrony if they were similar ages. In this case, the hatching was advanced or delayed depending on whether eggs were paired with older or younger counterparts. These results suggest that the *L. migratoria* eggs adjust the timing of hatching based on the information obtained from neighboring eggs, although the actual stimuli involved remain unknown.

Key words

egg mass, embryo-embryo communication, locust

Introduction

Many organisms lay eggs in masses, and these eggs are often observed hatching in synchrony (Colbert et al. 2010). A praying mantis is an example of an insect for which eggs hatch simultaneously from a pod (https://www.youtube.com/watch?v=0MV5mb0RJLY). Locusts are another example for which synchronous egg hatching occurs (Chen 1999, Nishide and Tanaka 2016). However, little is known about the underlying mechanism of the synchrony. Unlike some subsocial insects, in which egg hatching is facilitated by the parent (Ohba 2002, Mukai et al. 2012), most adult insects, including praying mantises and locusts, do not attend to their eggs. Therefore, the embryos in an egg mass likely control the timing of hatching by themselves, especially if synchronous hatching is important for their survival. The adaptive significance of hatching synchrony has been suggested in various animals; hatching synchrony may mitigate predation by diluting individual risk, swamping predators upon emergence, reducing cannibalism, helping hatchlings to form aggregations, or starting feeding in a group (Ghent 1960, Morimoto and Sato 1962, Arnold and Wassersug 1978, Dehn 1990).

Hatching time is influenced by a variety of factors during the embryonic stage. Many insects enter diapause at a species-specific embryonic stage (Tauber et al. 1986, Danks 1987). Embryonic diapause functions to synchronize hatching time with certain seasons and years. Any factor that affects embryonic development subsequently affects the hatching time (He et al. 2016). In addition, insects often use the surrounding temperature and photoperiod to control circadian rhythms or interval timers (Beck 1980, Saunders 2002). Eggs usually hatch at a certain time of day, and this time is often controlled by such mechanisms. Finally, there are finer, more sophisticated controls of hatching time. For example, water bugs hatch in synchrony from their egg mass in response to water sprayed by the male parent (Ohba 2002). In another example, the eggs of burrow and shield bugs hatch simultaneously in response to vibration produced by the female parent (Mukai et al. 2012, 2014).

Recently, the mechanisms controlling timing of hatching have been intensively studied in various species, mainly in vertebrates (Doody 2011, Spencer and Janzen 2011, Webster et al. 2015, Aubret et al. 2016, Cohen et al. 2016). In turtles, eggs are deposited in layers within a nest, and thermal gradients create optimal developmental conditions for each egg. Interestingly, the eggs hatch in synchrony through embryo-embryo communication, and each egg is able to adjust its individual development to match the rest of the nest (McGlashan et al. 2012). In red-eyed tree frogs, *Agalychnis callidryas*, eggs can hatch early to avoid predation by snakes and wasps in response to vibrational stimuli produced by the predators (Warkentin 1995, Warkentin et al. 2007). However, except for the few species of bugs mentioned above, the hatching synchrony of insects has been largely unexplored.

The migratory locust, *Locusta migratoria* L. (Orthoptera: Acrididae), is commonly found in grassland (Uvarov 1977). Its voltinism, or the number of generations per year, varies among geographic populations. The locust life cycle is univoltine in cool temperate regions, bivoltine or trivoltine in temperate regions, and multivoltine, with

more than 3 generations per year, in subtropical and tropical regions (Verdier 1972, Tanaka 1994a, b, Shimizu et al. 2012, Tanaka and Zhu 2008). Therefore, the hatching season varies among different geographic populations and among different generations at the same locality. Embryonic diapause plays an important role in controlling embryonic development to avoid hatching during adverse seasons; it allows eggs to hatch in favorable seasons (Tauber et al. 1986).

In *L. migratoria*, this coarse adjustment for hatching is controlled by environmental cues experienced by the eggs and their parents in bivoltine and trivoltine populations (Verdier 1972, Hakomori and Tanaka 1992, Tanaka 1992, Yamagishi and Tanaka 2009). Hatching within a more limited time frame is achieved by another mechanism. Laboratory studies suggest that egg hatching of *L. migratoria* occurs during the thermoperiod (high-temperature period) of thermocycles under continuous illumination, and during the light period of photocycles at constant temperature (Nishide et al. 2015a, b). The eggs of the eastern lubber grasshoppers, *Romalea microptera* (Smith et al. 2013) hatch during the thermoperiod in response to thermocycles. In *L. migratoria*, temperature and photoperiod are the main factors that cause the eggs to hatch during the 'right' period (Nishide et al. 2015a, b). However, the eggs do not always hatch in synchrony during the thermoperiod or light period. In contrast, in outdoor conditions, *L. migratoria* eggs hatch from 09:00 to 16:00 hours. However, the hatching for each pod lasts only a few hours (Chen 1999, Nishide et al. 2017). It appears that hatching synchrony depends on an additional mechanism, which is the main topic of the present study.

Hatching behavior has been investigated in detail for the desert locust, *Schistocerca gregaria* (Nishide et al. 2015a, b, Nishide and Tanaka 2016). Eggs kept in a mass were found to hatch in synchrony, while separated eggs did not hatch in synchrony. Furthermore, under constant temperature and illumination, eggs in a mass hatch earlier than those kept singly. These differences cannot be explained by temperature and light conditions alone. Hatching synchrony is also achieved in pairs of eggs if they are kept in physical contact with each other, but is not achieved if they are kept in separated containers or are located far apart in the same container. These observations indicate that tactile or vibrational stimuli may be involved (Nishide and Tanaka 2016), although the exact nature of the stimuli has not been identified. In the present study, I examine the hatching patterns of egg masses, the effects of egg mass size and egg separation on hatching, and the roles of egg masses in synchronizing hatching for *L. migratoria*.

Material and methods

Insects.—The *L. migratoria* strain used in this study originated in Okinawa, Japan. Numerous generations were maintained at 30°C and a 12 h photoperiod at the Tsukuba Laboratory of the National Institute of Agro-biological Institute at Ohwashi (NIASO). Eggs from this strain do not enter embryonic diapause at 30°C and hatch in 14–15 days. Adults were reared in a group of 100–200 individuals in wooden-framed cages (42 × 22 × 42 cm) covered with nylon screens. Egg pods were kept in moist river sand (ca. 15% moisture content) and held in plastic containers (diameter, 13 cm; height, 7.5 cm). Egg pods laid were washed with tap water within 24 h of deposition and held in clean moist sand in Petri dishes (9 × 2 cm) until use. The number of eggs in each pod varied greatly; only large egg pods containing 40–80 eggs were used in this study. Egg pods were incubated at 30°C under continuous illumination for different amounts of time per experiment protocol, and were washed once again to remove sand.

Hatching from egg pod.—To observe the pattern of hatching from egg pods, a female was allowed to lay an egg pod into moist sand that was filled to the top of a plastic PET bottle (volume, 280 ml) in a small wooden-framed cage (18 × 27 × 29 cm; Suppl. material 1A). The cardboard floor of the cage had a hole (~ 2 cm in diameter) through which the locust laid an egg pod into a sand-filled PET bottle (volume, 280 ml) placed underneath. The PET bottle containing an egg pod was capped and gently transferred to an incubator (Bio-Incubator, Fuji Ika Sangyo, Chiba, Japan) in which temperature was controlled at 30°C ± 0.2°C with continuous illumination. The test temperature was monitored with thermo-recorders (Ondotori TR-74Ui, T and D Co., Tokyo, Japan) every hour, and the light intensity was approximately 200 lx on the floor of each incubator.

On 2 days prior to expected hatching time, the cap was removed and a rectangular plastic case (14 × 18 × 2 cm) with a hole (2 cm diameter) in the center of the white-painted bottom was fixed on the top of the bottle with adhesive tape; this connected the mouth of the bottle to the hole of the case (Suppl. material 1B). The wall of the case was coated with Fluon to discourage climbing, and the case was covered with a transparent lid. In this apparatus, vermiform nymphs hatching from the eggs moved to the white floor and molted. In this study, hatching was quantified by counting the nymphs after this molt and the degree of synchrony was measured by measuring the time elapsed between the first and last hatching eggs in each pod or group. To record the number of hatchlings, a digital camera installed in an incubator was used to photograph the floor of the case every 30 min until no more hatchlings appeared, and the hatched nymphs in the photographs were counted later on a computer.

Effect of egg separation on hatching.—Six egg pods were washed and broken into two similarly-sized pieces on the day after deposition (day 1). For each pod, the eggs from one half were placed 1 or 2 mm away from one another on wet tissue paper in a plastic Petri dish (9 × 2 cm). The other pod half was wrapped in wet tissue paper and held in the same dish as the eggs from the first half, and kept far from the separated eggs.

On day 10, the separated eggs were transferred using fine forceps (W-29, Kowa Pincette, Tokyo, Japan) to another dish, where they were singly placed in small cone-shaped pits (4 × 10 mm) and were kept ca. 7 mm apart in moist white sand (15% moisture content; Brisbane White Sand, Hario Co. Ltd., Japan), and filled to a depth of 12–15 mm (10 pits per dish). Each egg was vertically placed with the anterior end upward. This was easily performed, as the posterior end displays a micropyle. Each of the pod halves containing eggs was placed horizontally and buried in sand in a dish (9 × 2 cm). To exclude possible differences in egg quality between different halves of the same pod, each treatment group consisted of three upper halves and three lower halves. The dishes containing these eggs were covered with a transparent lid and incubated at 30°C under continuous illumination. These dishes were photographed every 30 min to record the number of hatchlings, as described above. In the above experiment, the separated eggs experienced more handling disturbance than those kept in their pods. To minimize the handling disturbance, another experiment was carried out: all eggs were removed from their pods and divided into two groups on day 1. In one group, approximately 20 eggs were piled up as an egg mass on wet tissue paper in a Petri dish (n = 6), and 20 eggs from the other group were individually separated in the same dish. On day 10, the former eggs were transferred to another dish and were placed as an egg mass in a large sand pit

(10–12 × 10 mm). The eggs in the other group were singly placed in small sand pits in another dish as described above. The eggs were not covered with sand. Their hatching activity was recorded as described above. Because only those eggs that developed eyes by day 10 were used, hatchability was generally high (> 80%).

Effect of egg mass size on hatching.—Two experiments were carried out. In one experiment, all eggs from each of the 2–4 pods were incubated singly (isolation) or kept in groups of 2, 4, 10, or 20 on day 10 to determine the range of variation in hatching time. The eggs that were assigned to isolation were singly put in small sand pits in dishes (10 eggs per dish with no sand cover) as described above. Those assigned to small mass sizes (2 and 4) were buried in sand held in small transparent plastic dishes (3.5 × 1.5 cm), and those assigned to large mass sizes (10 and 20) were buried in sand in dishes (9 × 2 cm). The eggs were covered with a thin layer of moist sand (< 1 mm) to avoid desiccation. To minimize desiccation, the dishes containing eggs were kept in a plastic bag until day 13, when the monitoring of hatching activity started. The mean hatching time calculated for each egg pod was designated as 0 h, and the deviation from the mean was determined for all hatchlings. The data from different pods assigned to the same mass size were combined. The period from the beginning to end of hatching was also determined for each egg mass. The data for egg masses with a survival rate lower than 75% were discarded. The number of eggs analyzed ranged from 97 to 268. As mentioned, the eggs kept singly were not covered with sand. The sand cover tended to delay the time of their appearance from sand, as compared with eggs kept singly without sand cover (Suppl. material 2A; p < 0.05; t-test), whereas it did not significantly affect hatching time for eggs kept in pairs or in groups of 20 (Suppl. material 2B and C; p > 0.05; t-test each).

In the second experiment, the effect of egg mass size on hatching time was determined. Eggs from each pod were divided into two batches on day 10, and assigned to 2 mass sizes: 1 and 2, 2 and 4, 4 and 10, or 10 and 20. This was necessary, because hatching time was expected to vary from one egg pod to another, due to variation in time of oviposition (up to 24 h) and interpod variation, as will be described in the following section. The eggs were then handled as above, except for the 1 versus 2 egg treatment, in which eggs were placed in small pits with no sand cover in 24-well dishes to save space. The mean hatching time was separately determined for the two mass sizes from the same pod. Because preliminary observations indicated that eggs kept in larger mass groups tended to hatch earlier (not shown), the mean time for the larger mass group was designated as 0 h, and the relative hatching time for the smaller mass group was calculated. The data from different pods were combined, and the number of test eggs ranged from 72 to 174. Based on these results, the relative times of hatching in the different egg mass sizes were calculated by designating the mean hatching time for singly kept eggs as 0 h.

Effect of times of egg separation on hatching.—Eggs were obtained from 68 pods at different times after deposition. To minimize handling disturbance for eggs near hatching, the pods reaching day 10 were washed, and more than 40 eggs of each pod were piled up as a mass on wet tissue paper in a dish until used. A total of 20 eggs from each pod were placed as a mass in a large sand pit in a Petri dish as described above, and another 20 eggs from the same pod were singly held in small sand pits in another dish. The dishes containing eggs were then returned to the incubator where they were photographed to record the hatching activity.

In the 24 h before hatching, egg shells gradually become thinner and softer, making it difficult to handle eggs with a pair of forceps without damage. To minimize this problem, 29 pods were washed on day 10, and 48 eggs obtained from each pod were horizontally placed on moist sand as pairs in 24-well dishes. The eggs in each pair were placed in contact with each other. On day 13 or 14, the eggs of 12 pairs were separated from each other using a small paintbrush, and the other pairs in the same dish were similarly touched with the brush but without separation. Their hatching activity was recorded, as described above.

To determine if egg separation affected embryonic development, each of 4 pods was divided into two parts within 24 h after laying. The eggs from one part were individually separated, and those from the other part were kept in the pod on wet tissue paper in the same dish, as described above. On day 13, 20 eggs removed from these groups were transferred to different dishes where they were held singly in small sand pits (20 pits per dish). Hatching started 29.3 h later on average (range: 21–36.5 h). Hatching activity was recorded, as described above.

Effect of clumping of separated eggs on hatching.—Eggs from each of 42 pods were placed singly on wet tissue paper in a Petri dish on day 10. At different times before hatching, 20 eggs were kept as a mass in a large sand pit in a dish, and another 20 eggs were singly kept in small sand pits in another dish, as described above. They were not covered with sand. The hatching activity was recorded, as described above.

To keep the handling disturbance to a minimum, 38 egg pods were washed on day 10, and 48 eggs from each pod were placed horizontally as pairs on moist white sand in a 24-well plastic dish. The eggs in each pair were initially kept 2–3 mm apart. At different times before hatching, 12 pairs of eggs were put in contact with each other using a paintbrush, and the other pairs in the same dish were similarly touched with the brush but kept separated. The hatching activity was recorded, as described above.

Hatching synchrony between eggs from different pods.—To determine if eggs from different pods would hatch in synchrony, eggs obtained from two pods laid on the same or different days were held as pairs in contact with each other in small sand pits in 24-well dishes at days 9–12 when the older eggs reached day 12, and their hatching was recorded. Some pairs of eggs from each pod were continuously kept in contact with each other as controls. In another experiment, eggs were singly kept on wet tissue paper in a Petri dish at day 10 and were put together as pairs with younger eggs on day 12 in 24-well dishes. Some eggs from each pod were continuously kept singly as controls. To confirm the effect of pairing of isolated eggs at different ages on hatching using more pods, eggs from a pod were divided into 2 parts of similar sizes and handled as above. A total of 38 pairs of pods were used.

Statistical analyses.—The hatching times were compared using either Welch's t-test or Tukey's multiple comparison test. The comparisons of hatching intervals were made with the Steel-Dwass test. All tests were performed using a statistics service available at http://www.gen-info.osaka-u.ac.jp/MEPHAS/kaiseki.html or Statview (SAS Institute Inc., NC, USA).

Results

Patterns of hatching from egg pods.—*L. migratoria* eggs hatched after 14–15 days of incubation at 30°C. More than 50% of eggs hatched
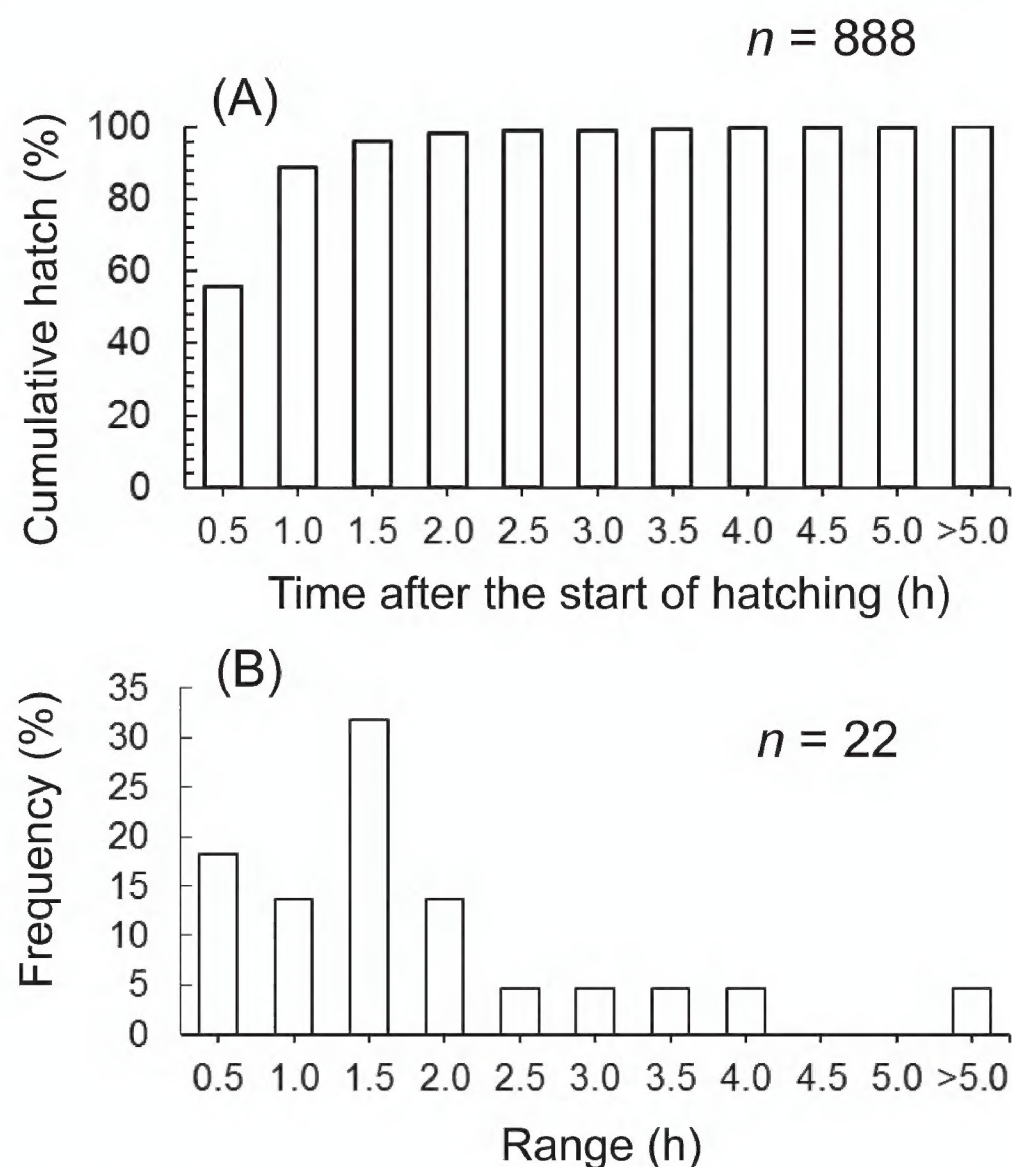
Fig. 1. Hatching patterns of *L. migratoria* eggs kept in the pods at 30°C under continuous illumination. Cumulative percentages of hatched eggs from 22 egg pods plotted against the time after the start of hatching for each pod (**A**). The frequency distribution of ranges from the beginning to end of hatching period from the respective pods (**B**).

from the egg pods during the first 30 min after the start of hatching at 30°C (n = 888 from 22 pods; Fig. 1A and Suppl. material 3). Most eggs (98.3%) hatched within 2 h. The length of the hatching period varied among the egg pods, with a mean of 2.4 h (n = 22; Fig. 1B). The most common hatching period was 1.5 h (31.8%), and 77.3% of egg pods completed hatching within 2 h.

Effect of egg separation on hatching.—The *L. migratoria* eggs that were removed from their egg pods on day 1 of laying and were singly placed in small pits in moist sand, hatched sporadically compared to those that were continuously kept in their egg pods. Fig. 2A-B compares these patterns of hatching by designating the mean hatching time in each group as 0 h. The eggs kept in the pods hatched across a period of 18 h (-6 to 12 h, Fig. 2A), whereas those kept singly hatched across a period of 68 h (-28 to 38 h, Fig. 2B). Because the latter eggs likely received more handling disturbance than the former eggs, another experiment was carried out: all eggs were removed from the pods at day 1 and incubated either singly or in a mass of 20 eggs. As shown in Fig. 2C, the eggs kept as egg masses hatched across a period of 4 h (0 to 4 h), whereas those kept singly hatched across a period of 86 h (-34 to 52 h; Fig. 2D).

Egg separation also affected hatching time. Fig. 2E and F illustrates the relative times required to hatch for eggs kept in either egg pods or 20-egg masses, or kept singly. This was done by designating the mean time for the egg pods or masses as 0 h. The eggs kept singly took significantly longer to hatch than those kept in pods (t-test; p < 0.01; Fig. 2E) or as masses (t-test; p < 0.01; Fig. 2F).

The effects of egg separation on the duration of the egg stage were also determined by collecting 8 egg pods within 1 h of laying (Fig. 3). The mean duration of the egg stage (± SD) for the individuals kept as masses of 20 eggs was 348.6 ± 10.7 h (range, 331–362 h; n = 8), whereas that for the singly kept eggs was 371.6 ± 16.9 h
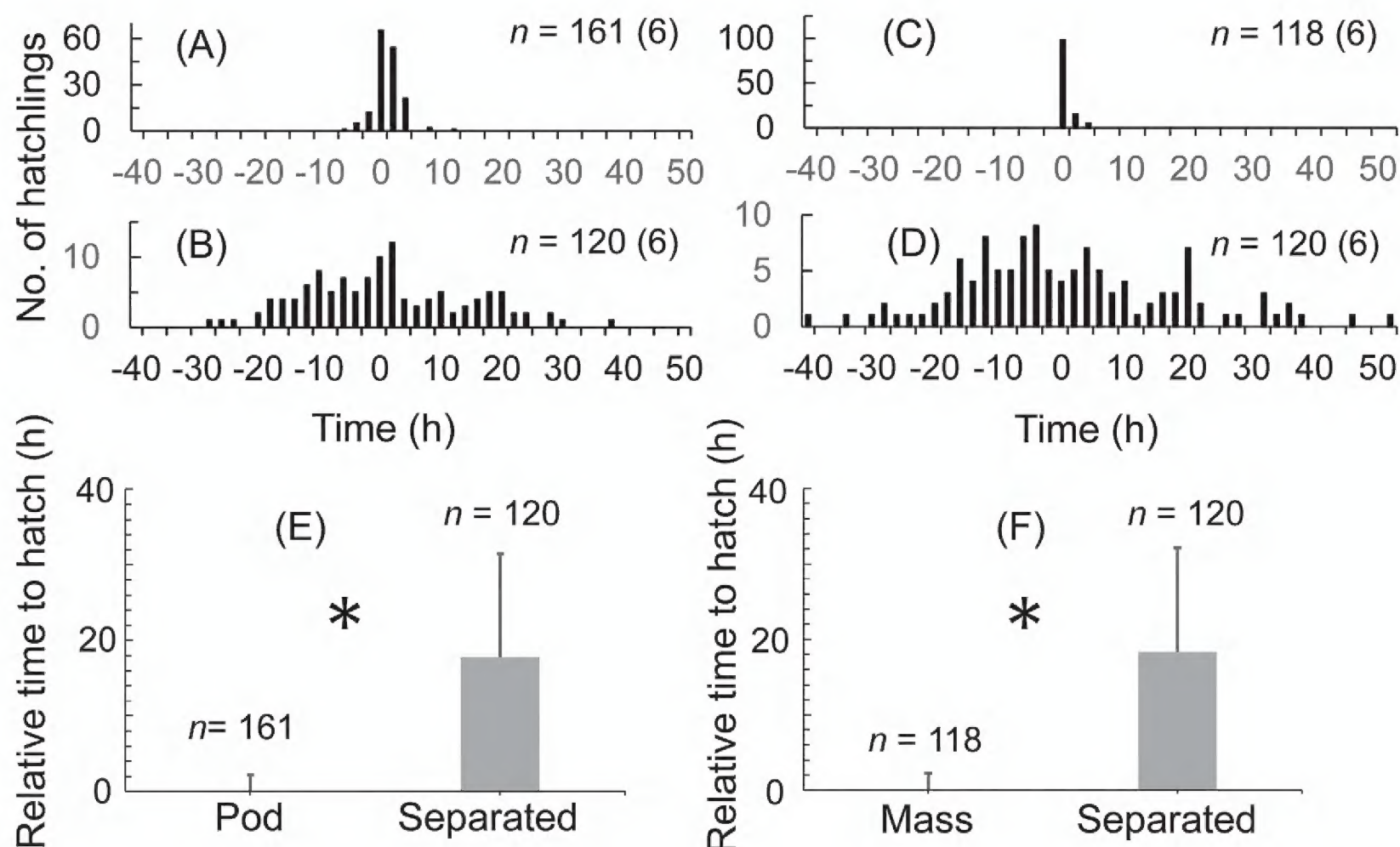

Fig. 2. The effects of egg separation on hatching patterns of *Locusta migratoria* eggs. The frequency distribution of hatching times for eggs kept in the pods (**A**) or in masses of 20 eggs (**C**) and those kept as separated eggs (**B, D**) when the mean hatching time was assumed to be 0 h. The relative times of hatching for the eggs kept in the pods (**E**) or in masses of 20 eggs (**F**) and those kept as separated eggs when the mean value for the former eggs was designated as 0 h. The number of hatched eggs followed by the number of tested pods in parentheses is given in each panel. Asterisks indicate a significant difference between the two treatments (p < 0.05; t-test).
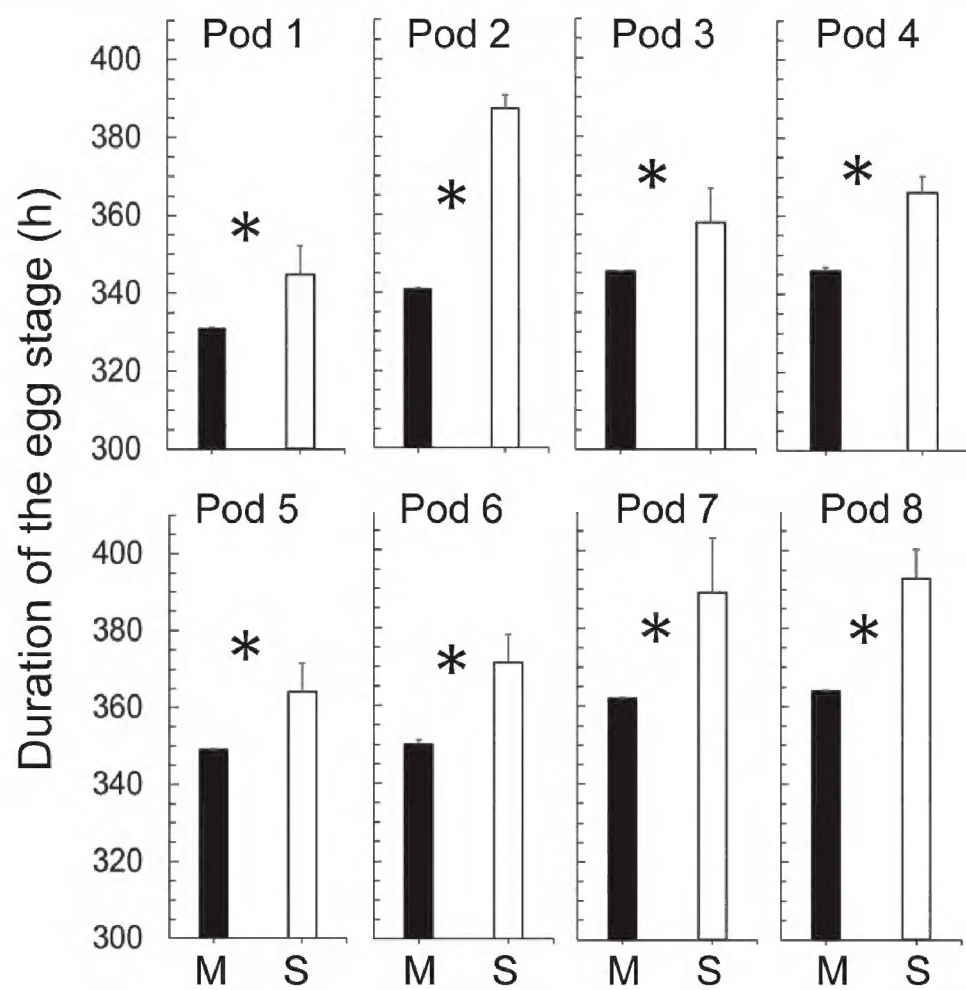
Fig. 3. The effect of egg separation on embryogenesis in *Locusta migratoria* at 30°C. The times (mean ± SD) required to hatch for *Locusta migratoria* eggs kept as a mass (closed bars) or as separated eggs (open bars) at 30°C. Asterisks indicate a significant difference between the 2 treatments at the 5% level with a t-test. Error bars indicate SD. n = 13–20 each.
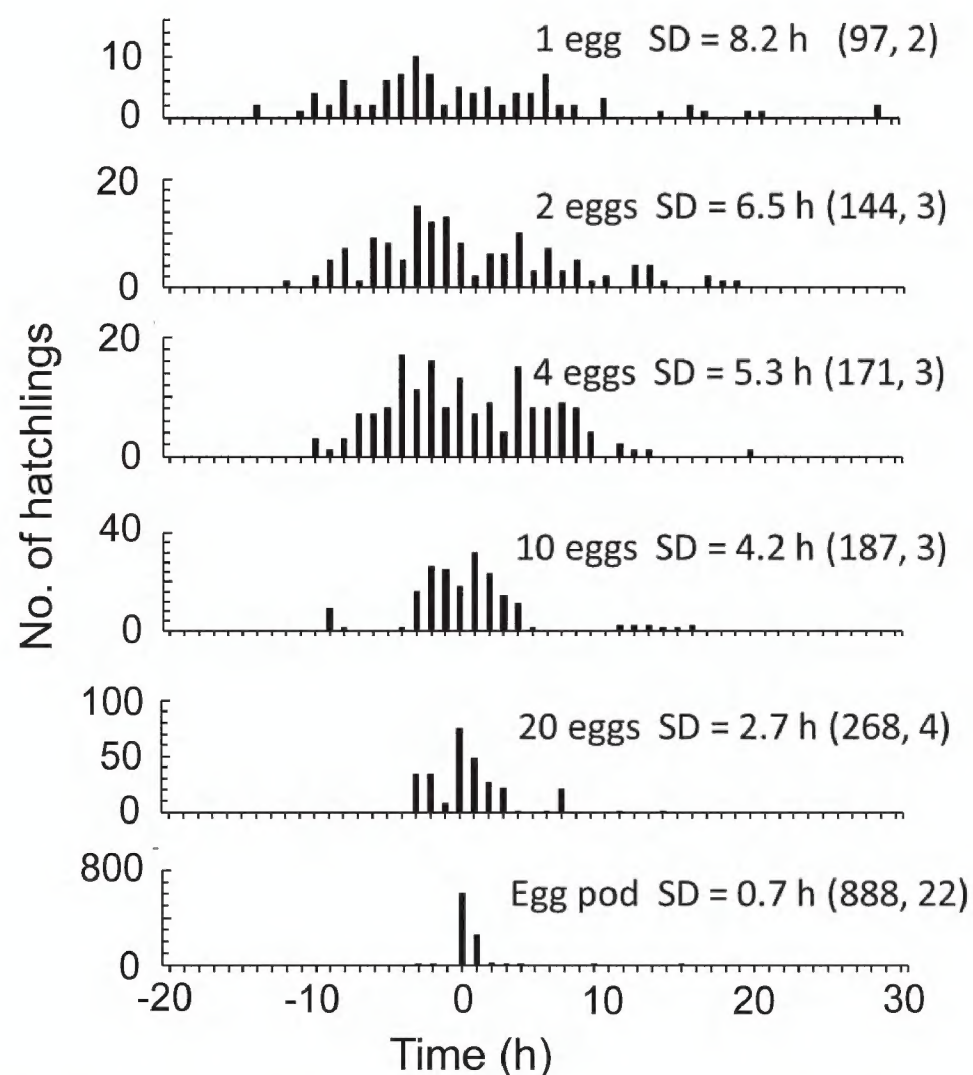

Fig. 4. The effect of egg mass sizes on the ranges of hatching times of *Locusta migratoria* eggs. The frequency distributions of hatching times plotted as deviations from the mean (designated as 0 h) for each egg pod tested. The data for the egg pods are the same as those given in Fig. 1B. SDs are shown. The numbers in parentheses indicate the numbers of hatched eggs and pods used.
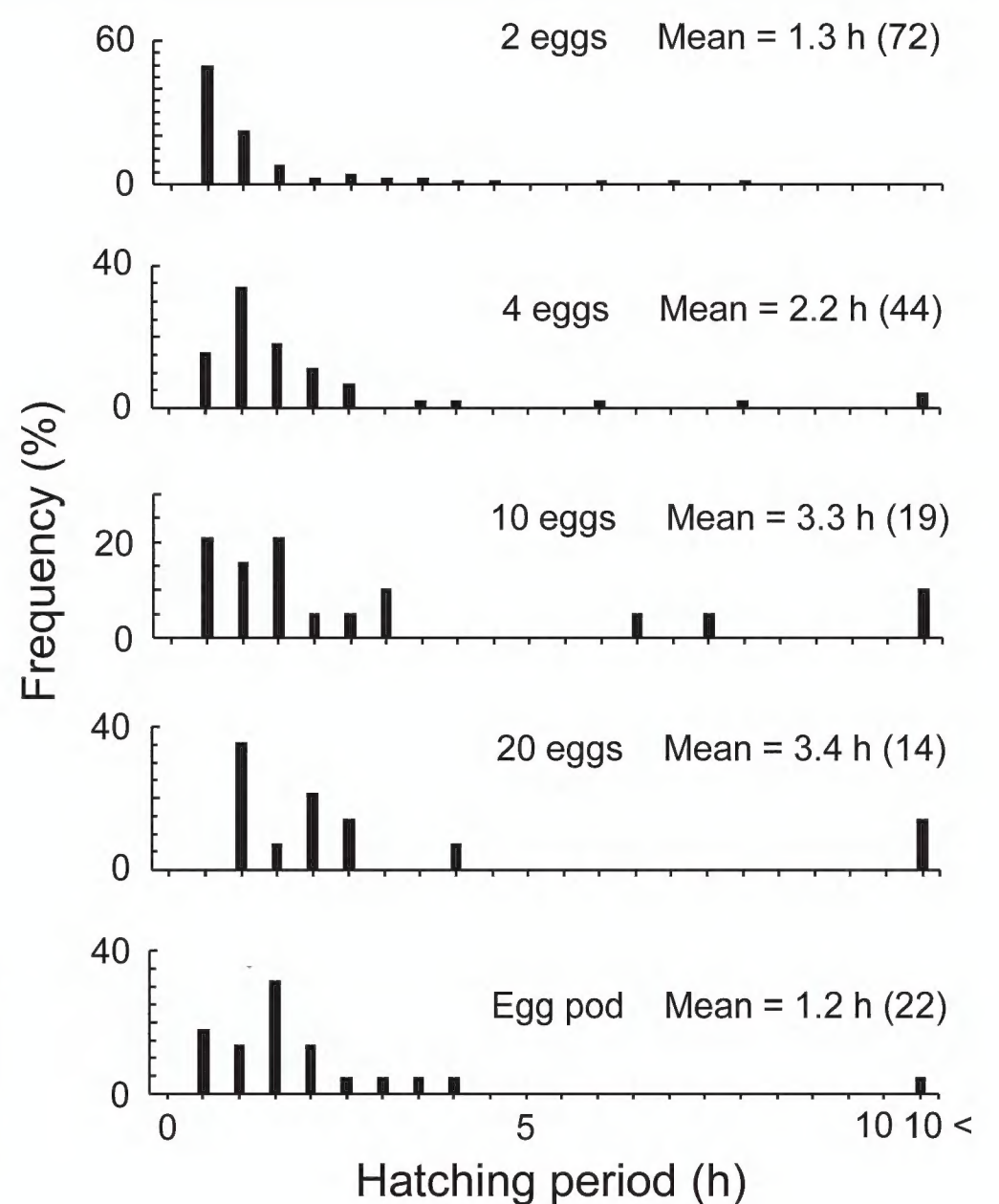
Fig. 5. The intervals from the beginning to end of hatching periods for *Locusta migratoria* eggs incubated in different mass sizes from day 10 onward. The data are based on the experiment described in Fig. 4. No significant difference was observed in the means among the treatments (p > 0.05; Steel-Dwass test).

(range, 358–392 h; n = 8), indicating that the mean duration of the egg stage at 30°C was approximately 14.5 days and 15.5 days, respectively. In all test pods, statistically significant differences were observed between the two groups (Fig. 3).

Effect of egg mass size on hatching.—Fig. 4 shows the distribution of hatching times for the eggs kept in various group sizes, calculated by designating the mean time for each egg mass size as 0 h. For comparison, the data for the eggs hatching from the pods shown in Fig. 1B are also included. The distribution of hatching times, as well as standard deviations (SDs) for the means, showed that the larger the egg mass size, the smaller the range of the hatching period for the whole group. In this experiment, the degrees of hatching synchrony for subgroups, as determined by the time elapsed between the first and last hatchlings for each dish or pod, was less than 4 h, irrespective of the differences in mass sizes (Fig. 5), and no significant differences were observed among the different mass sizes (p > 0.05; Steel-Dwass test).

Fig. 6 illustrates the relative hatching times for eggs kept in various mass sizes. In all pairs of comparison (Fig. 6A–D), hatching occurred significantly earlier in the larger mass group (p < 0.05 each; t-test). Based on these results, the relative hatching times were calculated by assuming that the mean hatching time for the eggs kept singly was 0 h (Fig. 6E). The difference in hatching times between single eggs and eggs kept in 20-egg masses amounted to approximately 20 h.
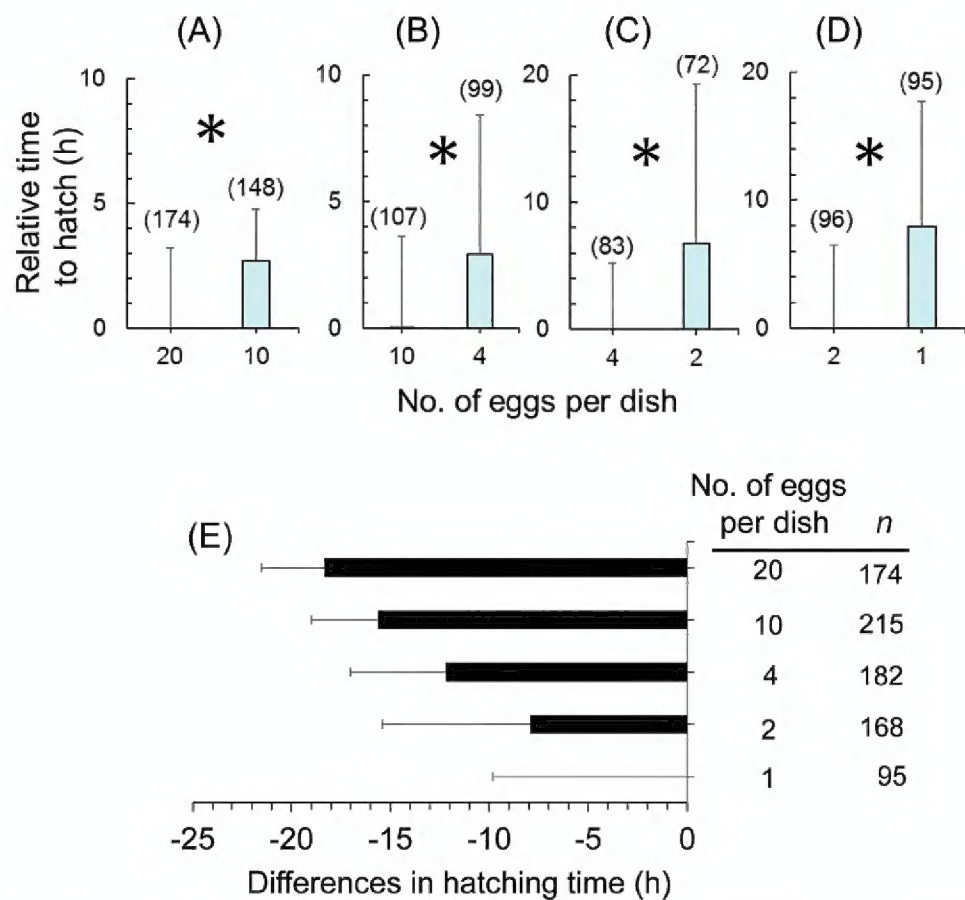
Fig. 6. The hatching times for *Locusta migratoria* eggs incubated in different mass sizes. In each pair of comparisons, the mean time of hatching for the eggs in the larger mass was designated as 0 h (**A–D**). The numbers in parentheses indicate the number of hatched eggs. Bars indicate one SD. Asterisks indicate a significant difference between the two treatments (p < 0.05; t-test). Differences in hatching time in different masses are shown in (**E**) by designating the mean hatching time for singly kept eggs as 0 h. Horizontal bars indicate one SD.
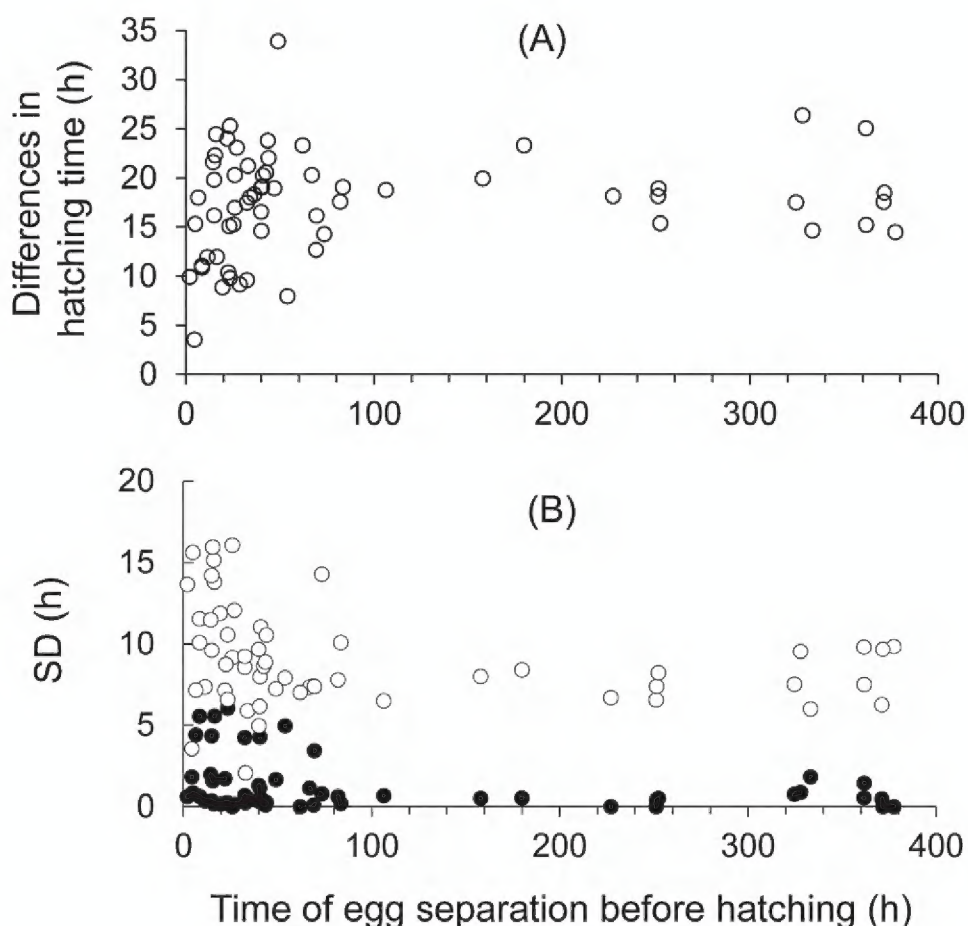
Fig. 7. The effect of the time of separation of *Locusta migratoria* eggs on the hatching time. The differences in mean hatching times between eggs separated and those kept in contact with each other (controls) are plotted against the time of egg separation gauged based on the mean hatching time for the control eggs kept in masses (**B**). Open and closed circles indicate eggs separated and those kept in masses, respectively. Each datum point is based on 16–20 eggs.
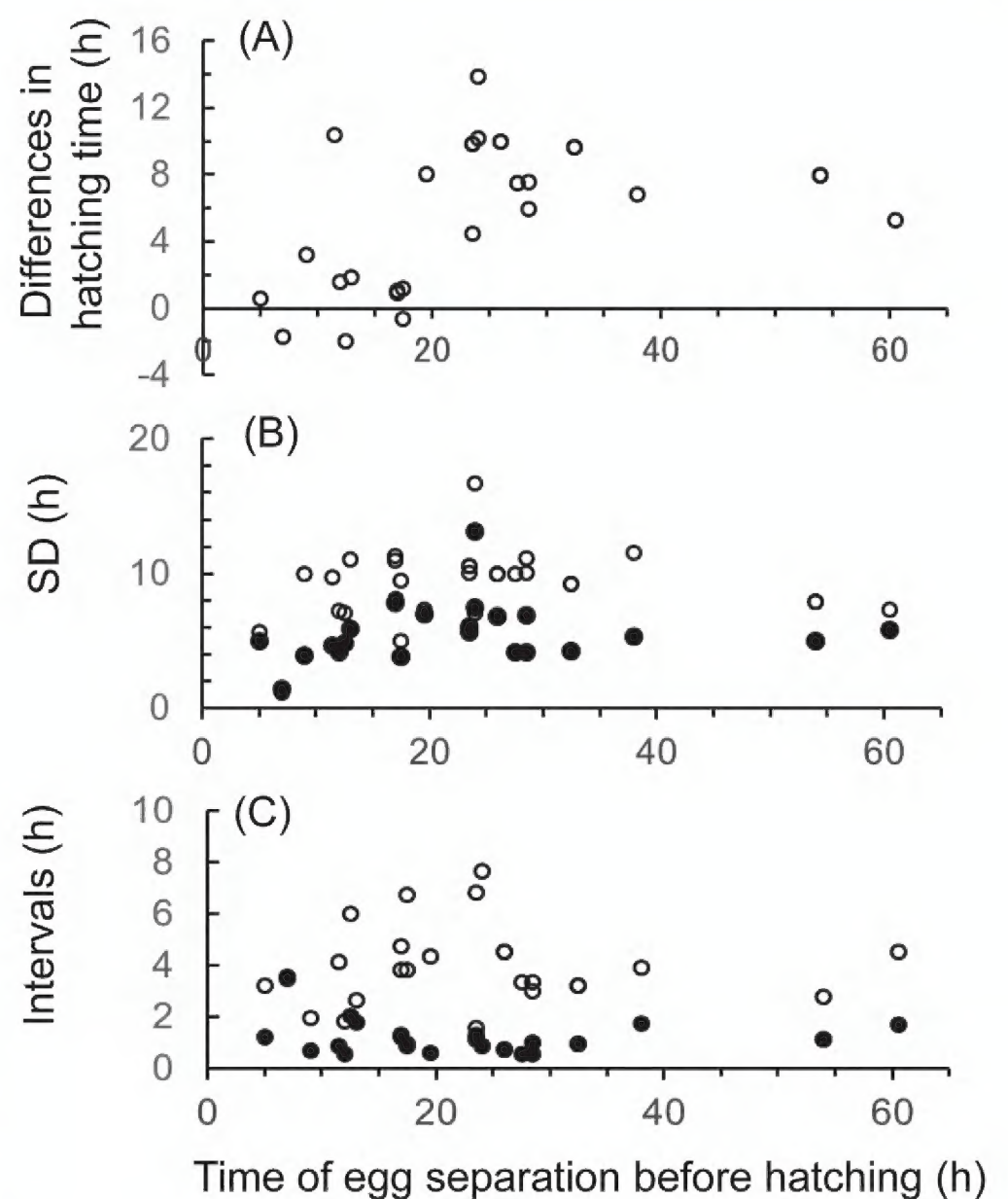

Fig. 8. The effect of the time of separation of paired *Locusta migratoria* eggs on the hatching time. The differences in mean hatching times between eggs separated and those kept in contact with each other (controls) are plotted against the time of egg separation gauged based on the mean hatching time for the control eggs (**A**). Comparison of SDs of the mean hatching times (**B**) and the intervals of hatching in each pair (**C**) for the eggs separated (open circles) and the control eggs (closed circles). Each datum point is based on 14–24 eggs.

Effect of the time of egg separation on hatching.—Each *L. migratoria* egg pod was divided into two groups at different occasions after laying and they were kept as separated eggs or as a mass. Fig. 7A shows the temporal variation in the differences in hatching times between the two treatments, plotted against the time of egg separation, and gauged based on the mean hatching time for the control eggs on the ordinate axis. The hatching of singly kept eggs was consistently delayed by 15 – 20 h compared to the control eggs that were kept as a mass until approximately 48 h before hatching (r = 0.06; df = 19; p > 0.05). During the last 48 h before hatching, a weak but significant positive correlation was found between the times of egg separation and the differences in hatching times (r = 0.26; df = 32; p < 0.05). The variation in hatching time was consistently larger for the singly kept eggs than for the eggs in masses, as indicated by the SDs (Fig. 7B). Relatively large SDs were observed for the eggs separated within 20 h before hatching. This increase in SD was likely due to handling effects.

To minimize the handling effects on hatching, eggs kept in contact with each other as pairs were separated with a paintbrush, and their hatching activity was compared with eggs that were continuously kept in contact with each other as controls. Fig. 8A shows the variation in hatching delay caused by egg separation plotted against
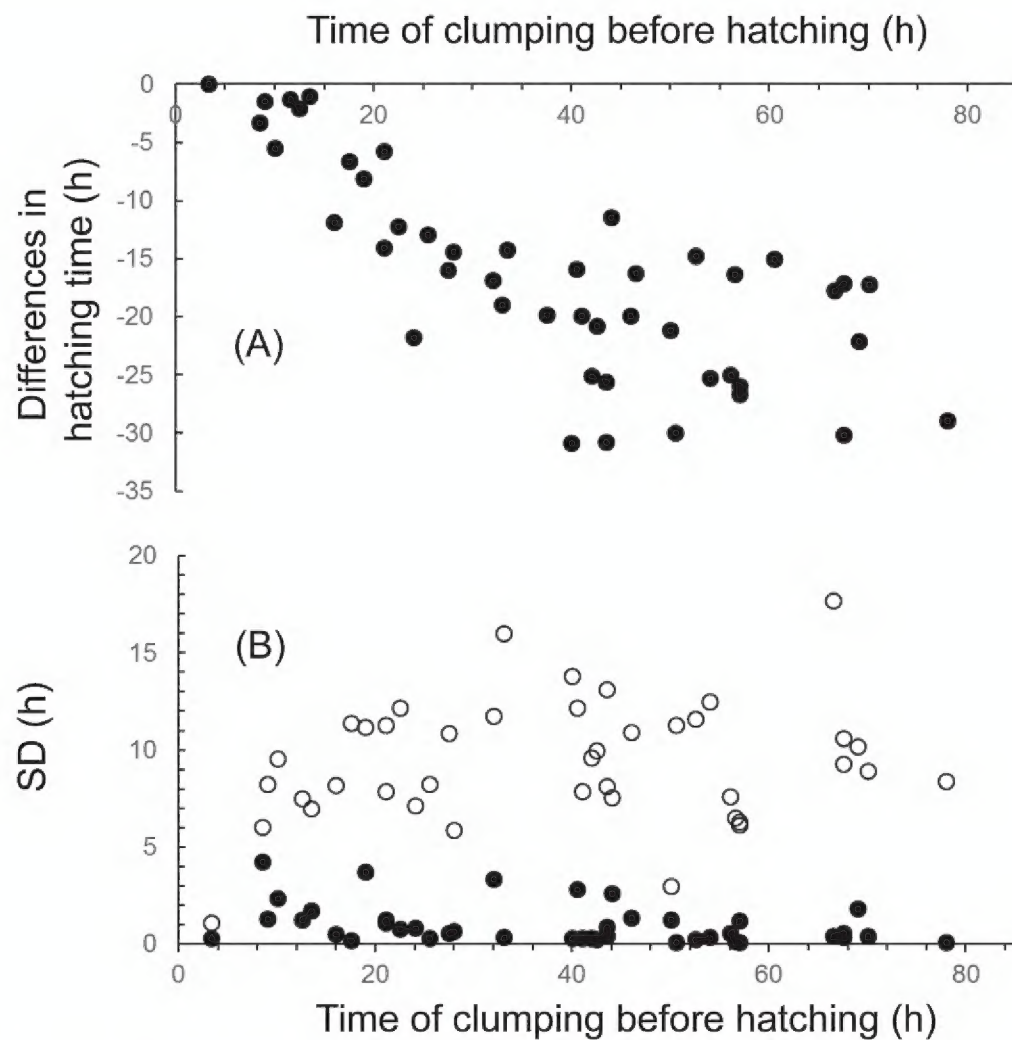
Fig. 9. The effect of the time of clumping of *Locusta migratoria* separated eggs on the hatching time. The differences in mean hatching times between eggs clumped and those kept with distance from one another (controls) are plotted against the time of egg clumping gauged based on the mean hatching time for the control eggs (**A**). SDs of the mean hatching times for the clumped eggs (closed circles) and control eggs (open circles) are similarly plotted in (**B**). Each datum point is based on 17–20 eggs.

the time of egg separation as described above. No significant correlation was observed between the two variables when the eggs were separated earlier than 25 h before hatching ($r = -0.47$; n = 11; p > 0.05). A significant correlation was observed when egg separation was performed within 24 h ($r = 0.64$; n = 17; p < 0.01). The regression analysis indicated that the differences in hatching times between the two treatments became 0 h when egg separation was performed approximately 7 h before the expected time of hatching for the control eggs ($Y = 0.49 \times - 3.60$; $R^2 = 0.41$; $F = 10.48$; df = 1, 15; p < 0.01). SDs were larger for the separated eggs than for those kept as masses until egg separation was performed at around 5 h before hatching (Fig. 8B). SDs gradually decreased as the egg separation approached hatching. Separation of eggs shortly before hatching, such as at 7 or 5 h, did not significantly affect hatching (t-test; p > 0.05), and both separated and clumped eggs took similar times to hatch (t-test; p < 0.05) with similar SDs. Egg separation increased the hatching intervals (the time elapsed between 2 hatchings) when performed 10 h or earlier before hatching (Fig. 8C).

The above results indicate that egg separation delayed hatching. To determine whether this delay was associated with delayed embryonic development or not, some eggs were separated within 24 h after laying, and others were kept in a mass until day 13, after which all eggs were kept singly until hatching. The mean time (± SD) required for the former to hatch after day 13 was 22.2 ± 9.5 h (n = 72) and that for the latter was 20.6 ± 11.8 h (n = 70), the difference being statistically insignificant (t-test; p > 0.05; Suppl. material 4), indicating that egg separation during the first 13 days of the egg period did not affect embryogenesis.
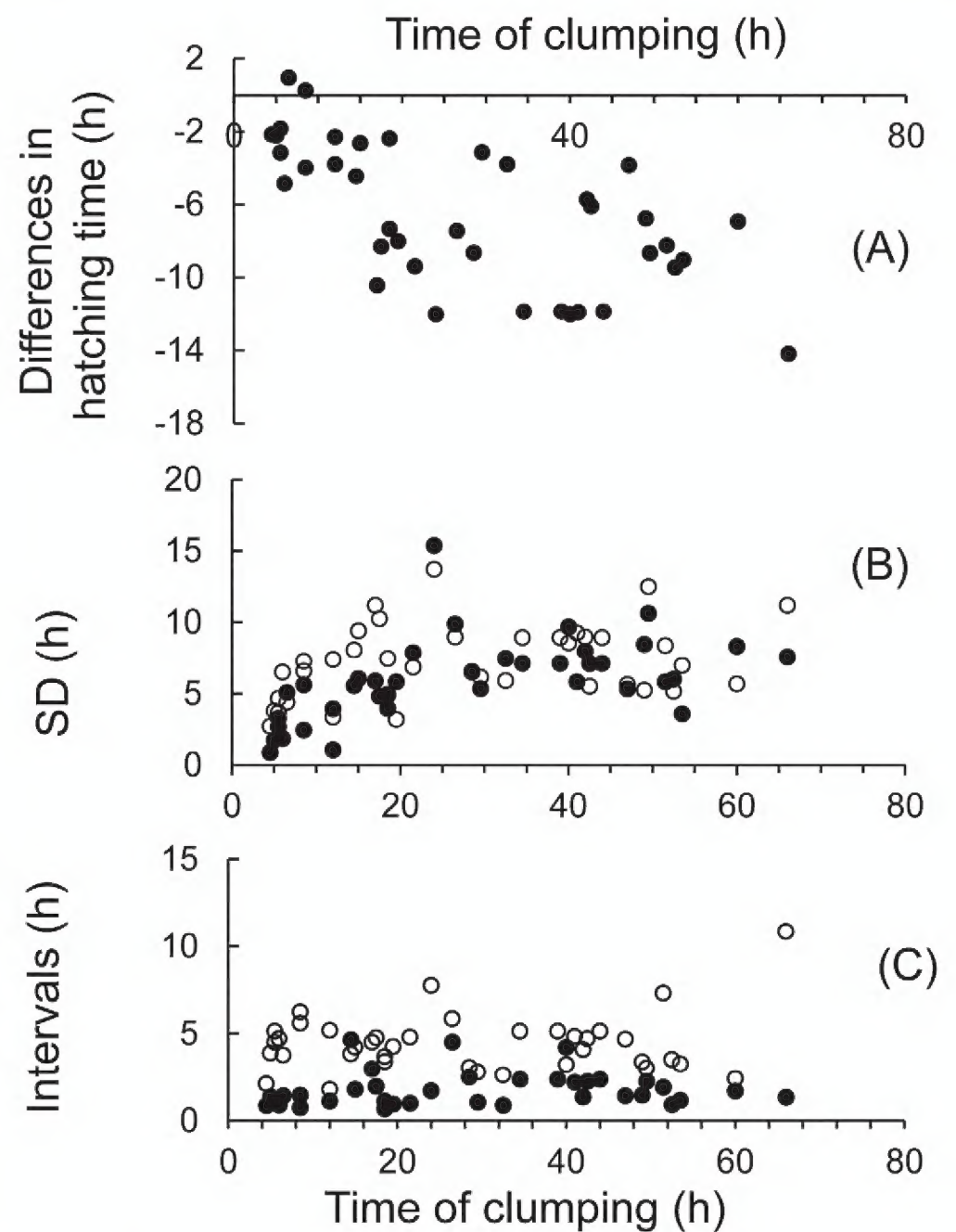

Fig. 10. The effect of the time of pairing of *Locusta migratoria* separated eggs on the time of hatching. The differences in hatching time between the paired eggs and control eggs kept as separated eggs are plotted against the time of egg clumping gauged based on the mean hatching time for the control eggs (**A**). Comparison of SDs of the mean hatching times (**B**) and the intervals of hatching in each pair (**C**) for the clumped eggs (closed circles) and the control eggs (open circles). Each datum point is based on 18–24 eggs.

Effect of clumping of isolated eggs on hatching.—Hatching was advanced when *L. migratoria* eggs separated on day 10 were clumped as a 20-egg mass at different times during the last several days of the egg stage as compared to those continuously kept separated until hatching (Fig. 9A). The differences in hatching times between the clumped and separated eggs ranged from 11.4 to 30.2 h when egg clumping in the former was performed greater than 40 h before the expected hatching time of the continuously separated eggs. The time of egg clumping did not significantly affect the differences in the mean hatching time between the two treatments ($r = 0.14$; n = 20; p > 0.05). When eggs were clumped within 40 h, in contrast, the differences in hatching times gradually decreased as the egg clumping approached hatching ($r = -0.88$; $Y = -0.72 \times + 4.72$; n = 15; p < 0.01). The regression line indicated that the difference became 0 h when egg clumping was performed 7 h before hatching. The differences in SDs between the two treatments remained more than 5 h until egg clumping was made within 6 h before hatching (Fig. 9B).

Similar results were obtained from *L. migratoria* eggs in pairs that were kept separated from each other on day 10 and were put together at different times before hatching. The eggs put together
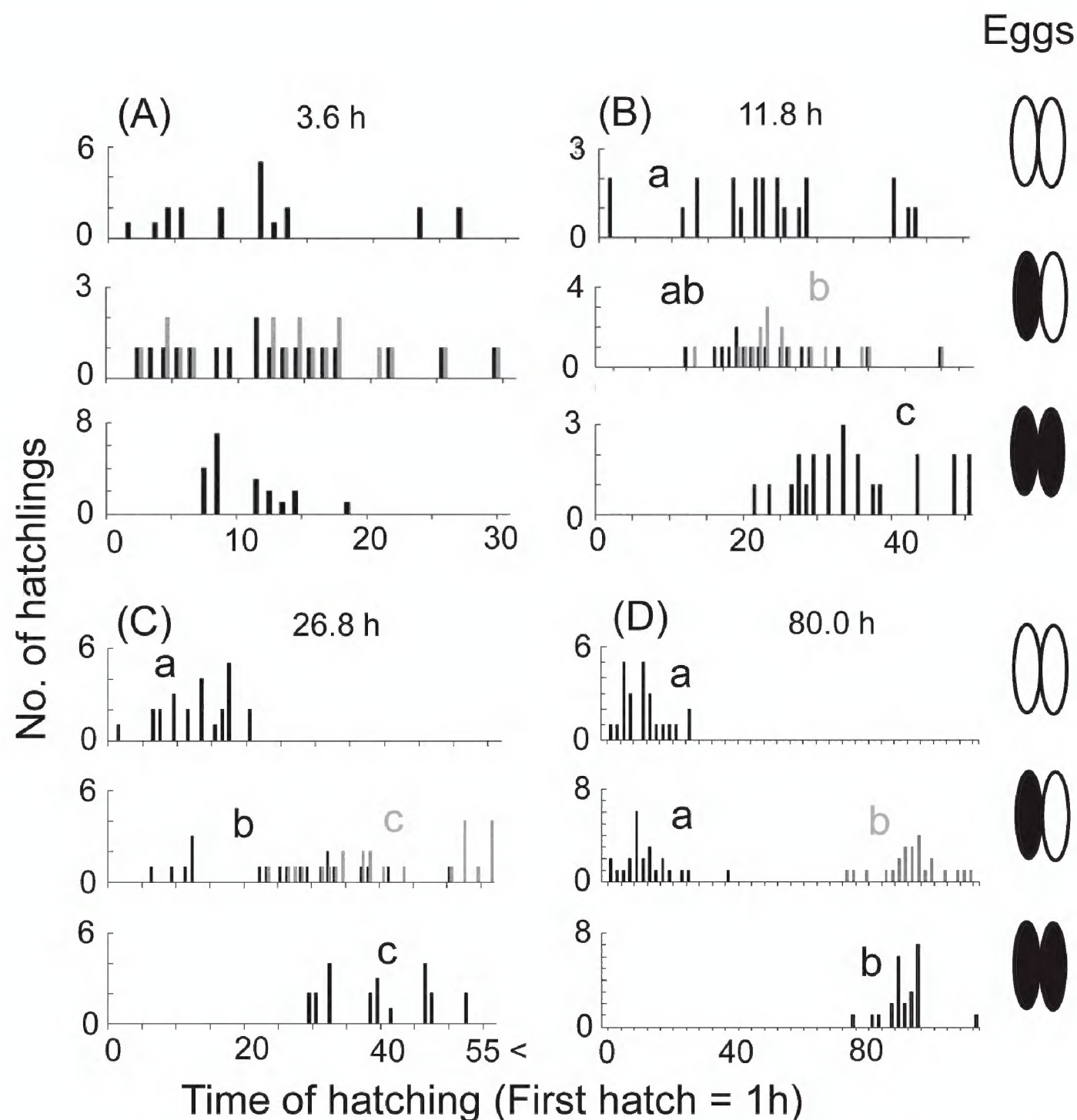
Fig. 11. Hatching patterns of *Locusta migratoria* eggs derived from different pods and those from the same pods. As indicated above each triplet, the difference in the mean hatching times of the 2 pods (top and bottom panels) ranged from 3.6 to 80 h (**A–D**). In the mixed pairs (middle panel), the first and second hatchings are shown in black and light-colored bars. Different lower-case letters indicate significant differences in mean values at the 5% level with Tukey's multiple test. The diagrams on the right of the figure show combinations of eggs from two pods expressed as white and black eggs, respectively.

hatched earlier than those continuously kept separated, and the differences in mean hatching time gradually decreased as the egg clumping approached hatching ($r = -0.77$; $Y = -0.44 \times + 0.94$; $n = 19$; $p < 0.001$; Fig. 10A). The regression line indicated that the difference became 0 h when egg clumping was performed approximately 2 h before hatching. The differences in SDs of the mean hatching times between the two treatments remained large until egg clumping was performed within 10 h before hatching (Fig. 10B). The hatching intervals of pairs of eggs were significantly reduced by clumping irrespective of its time (Fig. 10C), indicating that hatching synchrony was determined shortly before hatching.

Hatching synchrony of eggs from different egg pods.—The pairing of *L. migratoria* eggs obtained from two different pods showed three hatching patterns depending on how far the hatching times of the two pods were separated (Fig. 11). If the two pods were at similar ages (3.6 h apart; Fig. 11A), no significant differences were observed between the mixed pairs and the control pairs ($p > 0.05$; Fig. 11A). When two pods were produced 1–2 days apart, hatching time significantly differed between the two controls (top and bottom pan-

els in each triplet, Fig. 11B and C), and the mixed pairs showed an intermediate hatching time on average. In this case, the mean hatching times calculated separately for the first (black symbols) and second hatching eggs (light symbols) in the mixed pairs were significantly different from the value for the control eggs derived from the younger or older pod, indicating some interaction between the eggs in mixed pairs. When two pods produced 3 days apart were used (80 h; Fig. 11D), the eggs in the mixed pairs hatched either early or late. The first and second hatched eggs of the mixed pairs were apparently derived from the early- and late-produced pod, respectively. Fig. 12 shows the hatching intervals of pairs of eggs. No significant differences were observed in hatching intervals in each triplet when the two pods were produced 11.8 h apart or less (Steel-Dwass test; $p > 0.05$; Fig. 12A and B), indicating that eggs from different pods in the mixed pairs hatched in synchrony. As the difference in the mean hatching time between the two pods further increased, the mean hatching intervals for the mixed pairs became significantly longer than that for the control pairs (Fig. 12C and D).

Fig. 13 shows the results of experiments similar to the above experiments except that the control eggs derived from different pods were kept singly. The mixed pairs from two pods produced
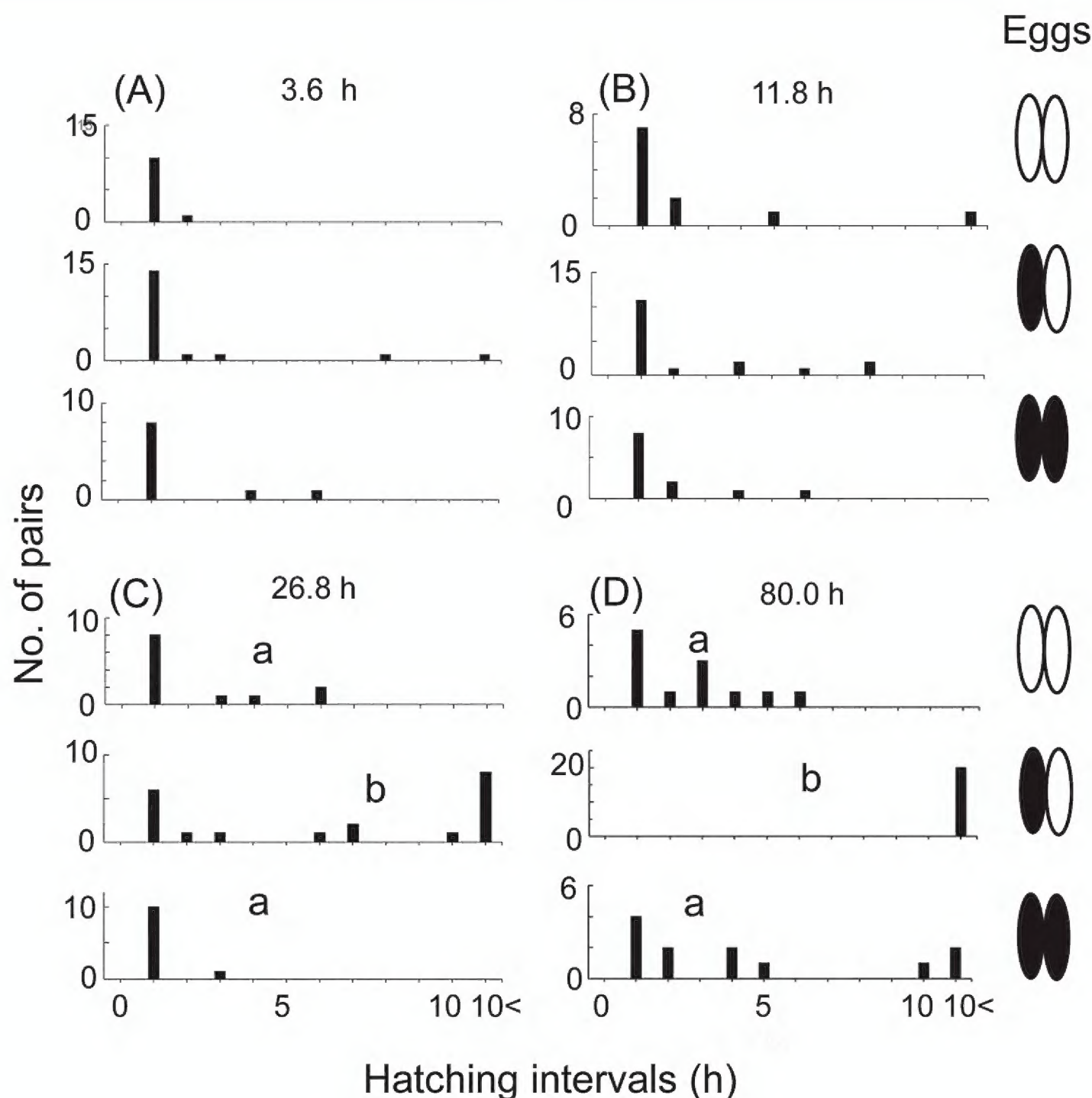
Fig. 12. The frequency distributions of hatching intervals for *Locusta migratoria* egg pairs derived from different (middle panel) and from the same pods (top and bottom panels). For explanation of the experimental design, see Fig. 11. Different lower-case letters indicate significant differences in mean values at the 5% level with Steel-Dwass test. The diagrams on the right of the figure show combinations of eggs from two pods expressed as white and black eggs, respectively.

on the same day hatched significantly earlier than the control eggs from each pod (Fig. 13A), as expected from Fig. 6D. They tended to hatch in synchrony with short hatching intervals (Fig. 13C). In contrast, when eggs were derived from 2 pods produced 1 day apart, the mean hatching time for the mixed pairs was intermediate between the values for the singly kept controls from the respective pods (Fig. 13B), indicating that singly kept eggs further delayed or advanced hatching, depending on the age of the egg paired. Most of the mixed pairs hatched in synchrony (Fig. 13D). This phenomenon was further analyzed using 38 pairs of pods produced on the same or different days (Fig. 14). The mean hatching intervals for the mixed pairs remained small when the two controls hatched 24 h apart or less (Fig. 14A), indicating that they adjusted the timings of hatching for synchronization. In contrast, when the differences in hatching times of the two controls increased to > 40 h, the hatching intervals of the mixed pairs increased accordingly ($r =$ 0.90; $p < 0.001$; n = 16), indicating little or no interaction between the two eggs. The first hatching eggs of the mixed pairs hatched significantly earlier or later than the early-produced (older) control eggs depending on the age of the eggs paired ($p < 0.05$; Fig. 14B). In contrast, their hatching time was not significantly modified if the two eggs were produced more than 40 h apart. The second hatching eggs, which were likely to have been derived from the late-produced (younger) pods, hatched significantly earlier than the late-produced control eggs if the two eggs were produced less than 60 h apart (Fig. 14C).

Discussion

L. migratoria eggs that live in grasslands hatch during the day from 0900 to 1600 (Nishide et al. 2017). Although the entire hatching period on each day is long, eggs hatch from each pod within 1–2 hours (Chen 1999, Nishide et al. 2017). These observations are consistent with the present study in the laboratory. Nishide et al. (2017) argue that hatching synchrony is important for the survival of hatchlings; predators, such as ants, attack locust hatchlings. A long hatching period is likely to increase the risk of predation by these predators.

The present study demonstrated that the mean hatching time for *L. migratoria* egg pods varied considerably, with a range of more than 30 h at 30°C under continuous illumination. As reported for *S. gregaria* (Nishide and Tanaka 2016), hatching was significantly delayed if the *L. migratoria* eggs were incubated singly, as compared to those kept in pods. Egg mass size also affected
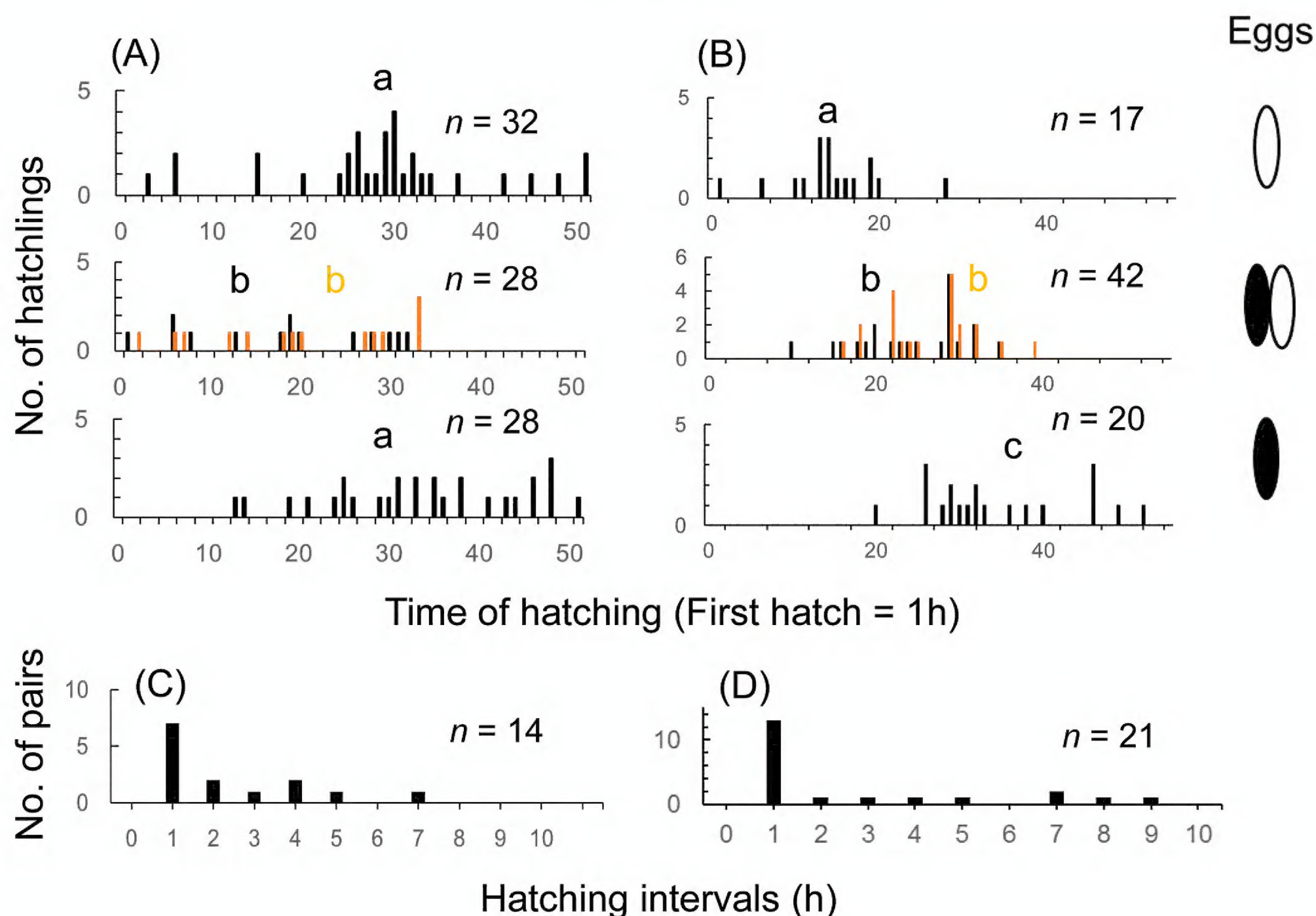
Fig. 13. Hatching patterns of *Locusta migratoria* eggs derived from different pods that were kept singly on day 10 and paired in contact with each other on day 12. Some eggs from the respective pods were singly kept as controls. Two pods laid on the same day (**A**) and different days (**B**) were used. In the mixed pairs (middle panel), the first and second hatchings are shown in black and light-colored bars. Different lower-case letters indicate significant differences in mean values at the 5% level with Tukey's multiple test. The diagrams on the right of the figure show combinations of eggs from two pods expressed as white and black eggs, respectively. The frequency distributions of hatching intervals for the mixed pairs are shown (**C** and **D**).

hatching time and its variance: the larger the egg mass, the earlier the hatching time, with a smaller variance. A similar phenomenon has been reported for the southern green stink bug, *Nezara viridula* (Kiritani 1964). In *L. migratoria*, the degree of hatching synchrony, measured as the time interval between the first and last hatching, was not significantly influenced by the mass size.

Artificial separation of eggs at different times after laying revealed that the differences in hatching time between eggs kept in a mass and those separated remained large until the last day before hatching. This was confirmed by another experiment, in which eggs were kept as pairs in contact with each other on day 10 and gently separated at different times before hatching. This method allowed us to observe the effects of egg separation on hatching activity with minimal handling. Similar results were obtained when the eggs individually separated on day 10 were clumped as egg masses at different times before hatching. The hatching intervals for the eggs clumped several hours before hatching were reduced to a small level compared to the values for the eggs continuously kept separated. These results suggest that the embryo of this locust controls the timing of hatching using stimuli derived from neighboring eggs shortly before hatching.

Hatching synchrony was observed in *L. migratoria* when eggs from different pods were put together as pairs. For hatching synchrony to be achieved, two pods should be at similar ages. Hatching activity of paired eggs was not influenced by the other if their ages were 3 days apart. Eggs at similar ages (< 2 days apart) can hatch in synchrony by adjusting their hatching time to the other, indicating that the stimuli involved in this phenomenon occur during the last 1 or 2 days.

At least three hypotheses explain the hatching behavior in *L. migratoria*. The first is that locust eggs wait for an appropriate signal from other eggs, so that singly kept eggs tend to delay hatching. They would become ready to hatch as soon as neighboring eggs start producing the stimuli. In the pods, eggs might be stimulated to hatch by early hatching individuals, resulting in synchronous hatching. This "passive" hypothesis may also explain that eggs in larger masses tended to hatch earlier, because the probability of receiving such stimuli earlier would increase as the egg mass is larger. However, it may not explain the phenomenon in which singly kept eggs further delay hatching if paired with a younger egg. This phenomenon might be explained by a second hypothesis that eggs control hatching time in response to stage-specific
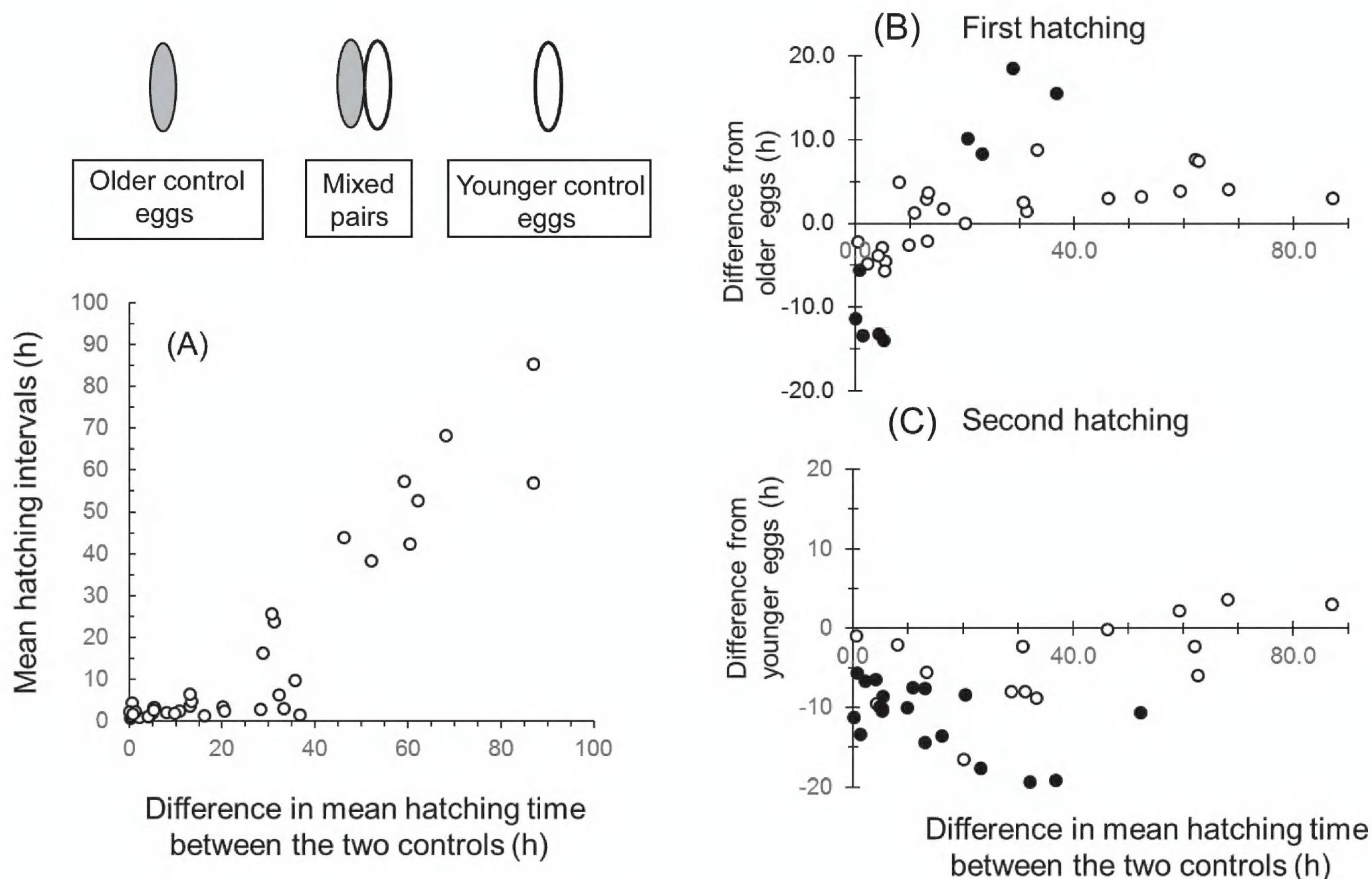
Fig. 14. The effects of pairing of *Locusta migratoria* eggs at different ages on hatching time. **A.** The mean hatching intervals between the first and second hatching eggs of the mixed pairs are plotted against the differences in mean hatching time between the younger and older controls in which two eggs were kept singly. **B.** The differences in hatching time between the first hatching eggs of the mixed pairs and older control eggs (from early-produced pods) are plotted against the difference in hatching time between the two control eggs. **C.** The differences in hatching time between the second hatching eggs of the mixed pairs and younger control eggs (from late-produced pods) are plotted against the difference in hatching time between the two controls. The diagrams on the top show combinations of eggs from older eggs (gray) and younger eggs (white). A total of 38 pairs of pods were used. Closed circles in (**B**) and (**C**) indicate the means significantly different from the controls (p < 0.05; Tukey's multiple test).

stimuli produced by neighboring eggs. The last hypothesis is that embryos communicate with one another to adjust the timing of hatching. The last two hypotheses explain equally well that singly kept eggs not only advanced, but also further delayed hatching, depending on whether they were paired with an older or younger egg. To determine which of the last two hypotheses is more likely, it is necessary to identify the stimuli used for synchronous hatching and to observe if eggs actually communicate with one another through such stimuli. Nevertheless, the present study documents that the *L. migratoria* eggs monitor neighboring eggs for synchronous hatching.

Embryo-embryo communication within a clutch is known in vertebrates, including birds and reptiles (Colbert et al. 2010, Doody 2011, Spencer et al. 2001, Vergne and Mathevon 2008, Vince 1966). In snakes, developing embryos use heart beats emanating from neighboring eggs as a clue for their metabolic level, to synchronize development and ultimately hatching (Aubret et al. 2016). Although such a mechanism may explain how the embryos adjust their development to one another, there might be an additional embryo-embryo communication signal to make the final

decision to hatch in synchrony. In birds and crocodiles, hatching has been suggested to be synchronized within a clutch through acoustic communication shortly before hatching (Woolf et al. 1976, Vergne and Mathevon 2008).

Costs involved in synchronous hatching are mainly discussed in association with shortened embryonic development (Colbert et al. 2010, Spencer and Janzen 2011, Warkentin 1995, Warkentin et al. 2007). In precocial birds, some individuals shorten incubation times for synchronous hatching, and show reduced motor skills (Vince and Chinn 1971, Cannon et al. 1986). In *L. migratoria*, however, embryonic development may not be modified by embryo-embryo interaction. Incubating eggs in a mass or isolation during the first 13 days of the egg stage made no significant difference in the hatching time if they were kept singly shortly before hatching (30 h on average; Suppl. material 4). As suggested for *S. gregaria* (Padgham 1981), locust embryos may enter a quiescent period after embryonic development and control the time of hatching. This idea is consistent with the present results that the clumping of separated eggs several hours before hatching caused them to hatch in synchrony.

For embryo-embryo communication in vertebrates, cues such as sound production, egg vibration, increase in heart rates, odors, or carbon dioxide levels within the nest have been proposed as potential communication signals (Spencer et al. 2001, Aubret et al. 2016). In *S. gregaria*, synchronous hatching is observed when the eggs are incubated in a mass, but not when separated (Nishide and Tanaka 2016). In this locust, hatching can be induced if the eggs are subjected to strong mechanical disturbance such as shaking or vortexing (Bernays1971, Nishide and Tanaka 2016), indicating that vibratory stimuli might be involved, although the relationship between this phenomenon and synchronous hatching is not clear. In *L. migratoria*, it is likely that stimuli transmitted through direct contact such as vibration are involved. This would be an interesting subject to be explored next.

Acknowledgements

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Supplementary material 1

Author: Seiji Tanaka

Data type: JPEG file

Explanation note: A cage to collect a *L. migratoria* egg pod (A) and an apparatus to observe hatching activity from an egg pod (B).

Link: https://doi.org/10.3897/jor.26.20935.suppl1

Supplementary material 2

Author: Seiji Tanaka

Data type: JPEG file

Explanation note: The effect of sand cover on the hatching time of *L. migratoria* eggs kept singly (A) and in masses (B and C). Eggs were placed singly or in masses in pits with or without sand cover at day 12. An asterisk indicates significant difference at the 5% level with a t-test. Error bars indicate SD. n.s. indicate no significant difference.

Link: https://doi.org/10.3897/jor.26.20935.suppl2

Supplementary material 3

Author: Seiji Tanaka

Data type: JPEG file

Explanation note: Photographs showing hatching of *L. migratoria* from egg pods recorded every 30 min at 30°C under continuous illumination.

Link: https://doi.org/10.3897/jor.26.20935.suppl3

Supplementary material 4

Author: Seiji Tanaka

Data type: JPEG file

Explanation note: The effect of egg separation on embryogenesis in *L. migratoria* at 30°C. Eggs of each pod were divided into 2 groups within 24 h after laying. The eggs in one group were kept as separated eggs (A) and those in the other group were kept in the pod (B) until day 13 when they were incubated individually until hatching. The 2 mean values were not significantly different from each other ($P = 0.35$; *t*-test).

Link: https://doi.org/10.3897/jor.26.20935.suppl4